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Stimulation of spinach (*Spinacia oleracea*) chloroplast fructose-1,6-bisphosphatase by mercuric ions

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Abstract Chloroplast fructose-1,6-bisphosphatase can exist in an active reduced form or a less active oxidised form. Oxidised fructose bisphosphatase from spinach (*Spinacia oleracea*) could be stimulated up to many hundred-fold by 0.1 mM $HgCl_2$ whereas fructose bisphosphatases from rabbit, yeast, a non-chloroplast enzyme from spinach and the reduced chloroplast enzyme were only inhibited by $HgCl_2$. Stimulation of the enzyme was maximal at pH 8.0 and low magnesium concentrations where the oxidised enzyme normally has little activity.

Key words: Chloroplast enzyme; Enzyme activation; Fructose-1,6-bisphosphatase; Mercury; Redox regulation; Spinach

1. Introduction

Chloroplast fructose-1,6-bisphosphatase, one of the enzymes of the photosynthetic carbon reduction cycle is activated when darkened leaves or chloroplasts are illuminated [1–5]. The purified enzyme exhibits several features that could account for light-dark regulation of the enzyme in vivo. The purified enzyme can exist in a reduced form or a less active oxidised form [6,7]. The enzyme can be activated in vitro by reduction, either chemically or by light-driven electron transport, while the activity of the enzyme – particularly the oxidised enzyme – can be increased considerably at high Ph and high magnesium ion concentrations. Since the stroma of the illuminated chloroplast is (more) reduced, has a higher Ph and magnesium ion concentration than in the dark it seems that the light activation found in vivo may consist of an as yet undefined combination of these effects.

A number of other enzymes of the chloroplast e.g. NADP-malate dehydrogenase and sedoheptulose bisphosphatase are light activated and in vitro exist in either a reduced active form or an inactive oxidised form [8,9]. These enzymes differ from the fructose bisphosphatase in that the oxidised enzyme possesses no detectable activity under physiological conditions. This difference in regulatory properties may reflect a need for the fructose bisphosphatase to be active to some extent in a less reducing environment. The recycling of hexose phosphates through the oxidative pentose phosphate pathway during metabolism of starch in the dark [10] may be just such a need. The light-dark regulation of chloroplast fructose bisphosphatase is clearly more complex than other light-activated enzymes and an understanding of the regulatory potential of the enzyme requires knowledge of the properties of both oxidised and reduced enzyme.

The results presented in this report describe the specific stimulation of oxidised chloroplast fructose bisphosphatase by mercuric ions and suggest possible explanations for the sigmoidal dependence of the enzyme activity on Mg^{2+} concentration.

2. Materials and methods

2.1. Materials

Chloroplast fructose-1,6-bisphosphatase was purified from spinach leaves to homogeneity (as judged by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate) by $(NH_4)_2SO_4$ and polyethylene glycol fractionation, DEAE-cellulose chromatography and gel filtration (A.R. Ashton, unpublished work). All steps were conducted in the absence of reducing agents. The purified enzyme has essentially the same physical and kinetic properties as the enzyme purified by others[6,7]. The non-chloroplast fructose-1,6-bisphosphatase was obtained from the fraction that was not retained by DEAE-cellulose during the purification of chloroplast fructose-1,6-bisphosphatase. Fructose-1,6-bisphosphatase from rabbit muscle, rabbit liver and torula yeast as well as the coupling enzymes phosphoglucose isomerase (yeast) and glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*) and other biochemicals were obtained from Sigma. The $HgCl_2$ was obtained from E. Merck.

2.2. Enzyme assay

Fructose-1,6-bisphosphatase activity was measured at 22° in a 1 ml reaction mixture containing 25 mM-Tricine {N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine}, 1 mM-fructose-1,6-bisphosphate, 0.5 mM-NAD, 2 mM-Mg $(NO_3)_2$, adjusted to pH 8.0 with KOH. Phosphoglucose isomerase (1 unit) and 1 unit of the glucose-6-phosphate dehydrogenase (which can utilise either NAD or NADP) were used as coupling enzymes. The coupling enzymes were not rate limiting in any of the experiments described here.

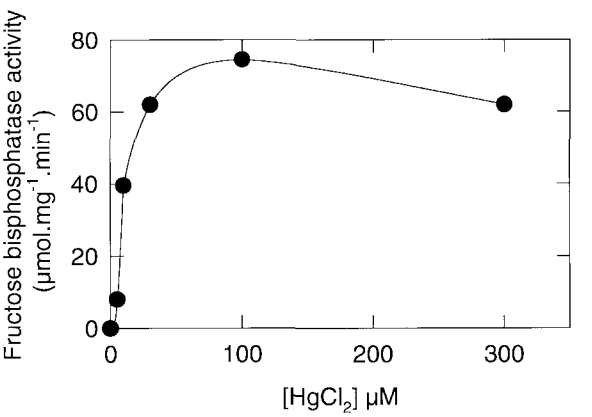

Fig. 1. Effect of $HgCl_2$ concentration on chloroplast fructose-1,6-bisphosphatase. Fructose-1,6-bisphosphatase activity was measured as described in Section 2 in assay mixtures containing 0.62 μg fructose-1,6-bisphosphatase and various concentrations of $HgCl_2$.

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Table 1
Effect of $HgCl_2$ on fructose-1,6-bisphosphatases from various sources

Enzyme	Fructose-1,6-bisphosphatase activity (μmol mg protein^{-1} min^{-1})		
	Control	+0.1 mM $HgCl_2$	Effect of $HgCl_2$
Spinach chloroplast	< 0.1	80.4	> 800-fold activation
Spinach non-chloroplast	0.69	0.35 (initial)	49% inhibition
		0.013 (after 5 min)	98% inhibition
Rabbit liver	1.51	0.21	86% inhibition
Rabbit muscle	0.19	0.04	79% inhibition
Yeast	24.4	21.6	11% inhibition

Fructose-1,6-bisphosphatase activity was measured as described in Section 2 at pH 8.0 with 2 mM $Mg(NO_3)_2$ and 0.1 mM fructose-1,6-bisphosphate except for the spinach non-chloroplast enzyme which was measured in the presence of 1 mM fructose-1,6-bisphosphate.

3. Results and discussion

The oxidised fructose-1,6-bisphosphatase of spinach chloroplasts possesses very little activity at pH 8.0 (or less) in the presence of 2 mM Mg^{2+}. The addition of 0.1 mM $HgCl_2$ to the enzyme results in a remarkable stimulation of activity (Table 1). This stimulation by $HgCl_2$ is specific for the chloroplast enzyme since fructose bisphosphatases from other sources are only inhibited by $HgCl_2$ (Table 1). The Hg(II) stimulation is also readily apparent in leaf extracts. A crude spinach leaf extract had a fructose bisphosphatase activity of 8 nmol mg protein^{-1} min^{-1} and 62 nmol mg protein^{-1} min^{-1} in the absence and presence of 0.1 mM $HgCl_2$, respectively. The Hg(II) stimulated fructose bisphosphatase demonstrable in crude extracts of spinach leaves copurifies to homogeneity with the Mg^{2+}-, high pH-stimulated and thiol-activated fructose bisphosphatase. The concentration dependence of the stimulation is shown in Fig. 1. Maximal stimulation is achieved at about 0.1 mM-$HgCl_2$ while at higher concentrations activity declines. The Hg(II)-stimulated fructose bisphosphatase gave a linear rate of fructose bisphosphate hydrolysis for at least 60 min at 22°. The Hg(II)-stimulation could be reversed during the course of the assay by addition of agents that sequester Hg(II), rapidly by 1 mM-dithiothreitol and more slowly by 1 mM EGTA plus 1 mM-$Mg(NO_3)_2$ ($t_{1/2} \sim 1$ min).

The effect of Hg(II) on the activity of the fructose bisphosphatase is very much dependent upon the assay conditions, as shown in Table 2. The greatest stimulation is achieved under conditions where the enzyme has little activity. The activity of the oxidised enzyme at pH 8.0 is increased ~100-fold by increasing the Mg^{2+} concentrations from 2 mM to 20 mM reflecting the sigmoidal dependence on Mg^{2+} concentration. The stimulation by Hg(II) is then only 2-fold. The requirement of the enzyme for Mg^{2+} cannot however be completely replaced by $HgCl_2$. Similarly by raising the pH from 8.0 to 8.9 (at 2 mM Mg^{2+}) the fructose bisphosphatase activity is increased several 100-fold but Hg(II) no longer stimulates the enzyme. Reduction of the enzyme also increases the fructose bisphosphatase activity several 100-fold when assayed at pH 8.0. Mercuric ions only inhibit the reduced fructose bisphosphatase although relatively weakly compared to other reduced light-activated enzymes such as NADP-malate dehydrogenase [8] or ribulose-5-phosphate kinase [11].

Although a thiol is the most likely candidate for a mercury-binding site, the native oxidised fructose bisphosphatase used here contains no thiol groups accessible to 5,5′-dithiobis (2-nitrobenzoate) (< 0.1 thiol per enzyme subunit). Moreover, 5,5′-dithiobis (2-nitrobenzoate) does not stimulate fructose bisphosphatase under conditions where Hg(II) stimulates the enzyme, nor does it prevent Hg(II) stimulation (Table 3).

Hg(II) was the only metal tested that could stimulate Mg^{2+}-dependent fructose bisphosphatase activity while not itself sustaining catalysis. Catalysis occurred in the presence of Mn^{2+}, Zn^{2+} and Co^{2+} alone while Pb^{2+}, Cd^{2+}, Ba^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+} (all 0.1 mM) did not stimulate the enzyme in the presence of 2 mM Mg^{2+}.

While mercury is usually regarded as an enzyme inhibitor it is not unknown for mercuric ions (or more commonly organic mercurials) to stimulate enzymes [12]. Frequently the stimula-

Table 2
Effect of $HgCl_2$ on the activity of oxidised and reduced chloroplast fructose-1,6-bisphosphatase

Fructose-1,6-bisphosphatase		Fructose-1,6-bisphosphatase activity (μmol mg protein^{-1} min^{-1})			
	pH	[Mg^{2+}]	Control	+0.1 mM $HgCl_2$	Effect of $HgCl_2$
Oxidised	8.0	0	0.0	0.0	No effect
Oxidised	8.0	2	0.2	55.9	280-fold activation
Oxidised	8.0	20	26.5	55.3	2.1-fold activation
Oxidised	8.9	0	0.0	0.0	No effect
Oxidised	8.9	2	70.0	61.6	12% inhibition
Oxidised	8.9	20	68.2	50.2	26% inhibition
Reduced	8.0	2	69.3	59.1	15% inhibition
Reduced	8.0	20	64.6	54.9	15% inhibition

Fructose-1,6-bisphosphatase activity was measured as described in Section 2 either at pH 8.0 or pH 8.9 in the presence of 0.1 mM $HgCl_2$. Assays contained 0.82 μg of oxidised fructose bisphosphatase or 0.65 μg of reduced fructose bisphosphatase. Reduced fructose bisphosphatase was obtained by incubating the fructose bisphosphatase in 100 mM Tricine pH 8.0 containing 25 mM dithiothreitol, 2 mM Ca $(NO_3)_2$, 1 mM fructose-1,6-bisphosphate at 4° for 24 h. The presence of Ca^{2+} and fructose bisphosphate, which presumably act as a non-hydrolysable substrate analogous, markedly enhances the rate of enzyme reduction [18]. The reduced fructose bisphosphatase was desalted by passage through a Sephadex G-25 column equilibrated with 50 mM acetate (Na^+) pH 6.0 containing 1 mM dithiothreitol. Aliquots of 5 μl were used in 1 ml assay mixtures which thus contain 5 μM dithiothreitol. This dithiothreitol would sequester 10% of the added Hg(II).

Table 3
Effect of 5,5-dithiobis (2-nitrobenzoate) on fructose-1,6-bisphosphatase activity

Assay condition	Fructose-1,6-bisphosphatase activity (μmol mg^{-1} min^{-1})		
	1 min	10 min	+$HgCl_2$
pH 8.0	0.26	0.26	28.2
pH 8.0 +0.1 mM 5,5-dithiobis (2-nitrobenzoate)	0.06	0.16	23.2

Fructose-1,6-bisphosphatase activity was followed as described in Section 2 in the presence or absence of 0.1 mM 5,5-dithiobis (2-nitrobenzoate) for 10 min and then 0.1 mM $HgCl_2$ was added to the assay and the activity found 1 min later was recorded.

tion involves release of some sort of inhibitory constraint as in the case of aspartate transcarbamylase where organic mercurials dissociate the regulatory subunits from the catalytic subunits [13]. However, stimulations of the extent found here for fructose-1.6-bisphosphatase have not previously been described. The role of mercury in stimulating oxidised fructose bisphosphatase seems to be to satisfy the need for high Mg^{2+} concentrations but not to completely replace the Mg^{2+} requirement. The sequence homology between spinach chloroplast fructose bisphosphatase and the cytoplasmic fructose bisphosphatase from pig kidney [14] and the more recent crystal structure determinations [15,16] suggest a possible mechanism for the Hg(II)-dependent stimulation. Animal fructose bisphosphatases possess at least two metal binding sites per subunit, with two sites being associated with the binding of the substrate fructose-1,6-bisphosphate [15–17]. These sites can be occupied by Mg^{2+}, Mn^{2+}, Co^{2+} or Zn^{2+} in a catalytically competent enzyme. Thus a possible explanation for the Hg(II) stimulation of chloroplast fructose bisphosphatase is that at pH 8 or less one of the metal binding sites binds Mg^{2+} only weakly but can bind Hg(II) at low concentrations. The Mg^{2+} bound at the other metal binding site however cannot be replaced by Hg(II). Presumably at higher pH or when the enzyme is reduced this weaker binding site can bind Mg^{2+} more tightly. When this site is occupied by Mg^{2+} (i.e. at high pH or pH 8 with high Mg^{2+} concentration or for the reduced enzyme at pH 8 with low Mg^{2+} concentration) stimulation by Hg(II) is minimal. Such a dual requirement for Mg^{2+} at two metal ion binding sites could help explain the sigmoidal dependence of the activity of oxidised fructose-1,6-bisphosphatase on the Mg^{2+} concentration.

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